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# Differentiation of Appendiceal Carcinoids By Marker Gene Expression

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# **Differentiation of Appendiceal Carcinoids**

## **By Marker Gene Expression**

**Igor Latic**

**Yale School of Medicine**

**2006**

**MD Thesis in partial fulfillment of graduation requirement**

### **Acknowledgements:**

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Supported by: Office of Student Research, YSM - NIH Cancer Education Grant/Yale Cancer Center Medical Student Research Fellowship; Department of Surgery, YSM - Charles Ohse Grant for Surgical Research.

Specific Contributions:

*Tissue collection:* Igor Latic

*RNA Isolation:* Mark Kidd, Igor Latic

*Q RT-PCR:* Mark Kidd, Igor Latic, Shrikant Mane

*Immunostaining:* Mark Kidd, Igor Latic, Robert Camp

*Statistical analysis:* Mark Kidd

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**Abstract**

**Objective:** To utilize differential gene expression of candidate markers to discriminate benign APCs (APCs) from malignant and mixed cell APCs.

**Background Data:** Considerable controversy exists in regard to the appropriate surgical management of APCs since standard clinical and immunohistochemical methods cannot reliably determine whether an APC is indolent or aggressive. We have identified five differentially expressed genes: nucleosome assembly protein 1-like 1 [*NAP1L1*]; melanoma antigen D2 [*MAGE-D2*]; metastasis-associated protein 1 [*MTA1*]; NAcH Leucine-rich-repeat Protein 1 [*NALP1*] and Chromogranin A [*CgA*] that define gut neuroendocrine (NE) Enterochromaffin (EC) cell behavior. We hypothesized that APC (APC) malignancy, also derived from EC cells, could be defined by using quantitative reverse transcriptase-polymerase chain reaction (QRT-PCR) and immunohistochemical approaches that evaluate potential marker genes.

**Methods:** Total RNA was isolated using TRIzol reagent from 42 appendiceal samples including APCs identified at exploration for appendicitis (no evidence of metastasis;  $n=16$ ), appendicitis specimens ( $n=11$ ), malignant appendiceal tumors ( $>1.5\text{cm}$ , evidence of metastatic invasion;  $n=7$ ) and mixed (goblet) cell appendiceal (GBC) adenocarcinoids ( $n=3$ ), normal appendiceal tissue ( $n=5$ ), and five colorectal cancers. Gene expression (*CgA*, *NAP1L1*, *MAGE-D2*, *MTA1* and *NALP1*) was examined by Q-RT PCR (Applied Biosystems) and quantified against *GAPDH*.

**Results:** *CgA* message was elevated ( $>1000$ -fold,  $p<0.05$ ) in all tumor types. *NAP1L1* was elevated ( $>10$ -fold,  $p<0.03$ ) in both malignant and GBC adenocarcinoids compared to normal and incidental lesions ( $p<0.006$ ). *MAGE-D2* and *MTA1* message were

significantly elevated ( $>10$ -fold,  $p<0.01$ ) in the malignant and GBC adenocarcinoid tumors but not in the appendicitis-associated carcinoids or normal mucosa. The apoptotic marker, *NALP1*, was over-expressed ( $>50$ -fold,  $p<0.05$ ) in the appendicitis-associated and malignant APCs, but was significantly decreased ( $>10$ -fold,  $p<0.05$ ) in GBC adenocarcinoids. Elevated *CgA* levels indicative of a carcinoid tumor were identified in one acute appendicitis sample with no histological evidence of a tumor.

**Conclusions:** These data demonstrate that malignant APCs and GBC adenocarcinoids have elevated expression of *NAP1L1* (mitotic regulation), *MAGE-D2* (adhesion), and *MTA1* (estrogen antagonism) compared to APCs identified at appendicitis. This, and the differences in *NALP1* (apoptosis) gene expression (decreased in GBC adenocarcinoids), provides a series of molecular signatures that differentiate carcinoids of the appendix. *CgA* identified all appendiceal tumors as well as covert lesions, which may be more prevalent than previously recognized. The molecular delineation of malignant appendiceal tumor potential provides a scientific basis to define the appropriate surgical management as opposed to morphological assessment alone.

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## ***1. Introduction***

Although generally regarded as an insignificant organ, the appendix at the turn of the 19<sup>th</sup> century was a source of considerable vexation to clinicians since the diagnosis of appendicitis was often difficult and outcome commonly associated with considerable morbidity and mortality.<sup>1</sup> It is of interest that a century later, although the problem of appendicitis has dramatically receded in the pantheon of medical problems, the pathological delineation and management of appendiceal tumors, particularly carcinoids remains an area of confusion and difficulty.<sup>2-4</sup> The histology of the tumor is often equivocal, the identification of microscopic spread often difficult to identify if infection is present, and management decisions are often made on an empiric or purely judgmental basis. This investigation seeks to identify a molecular profile that can define appendiceal malignancy and be utilized to provide a basis for the development of rational surgical and oncological management.

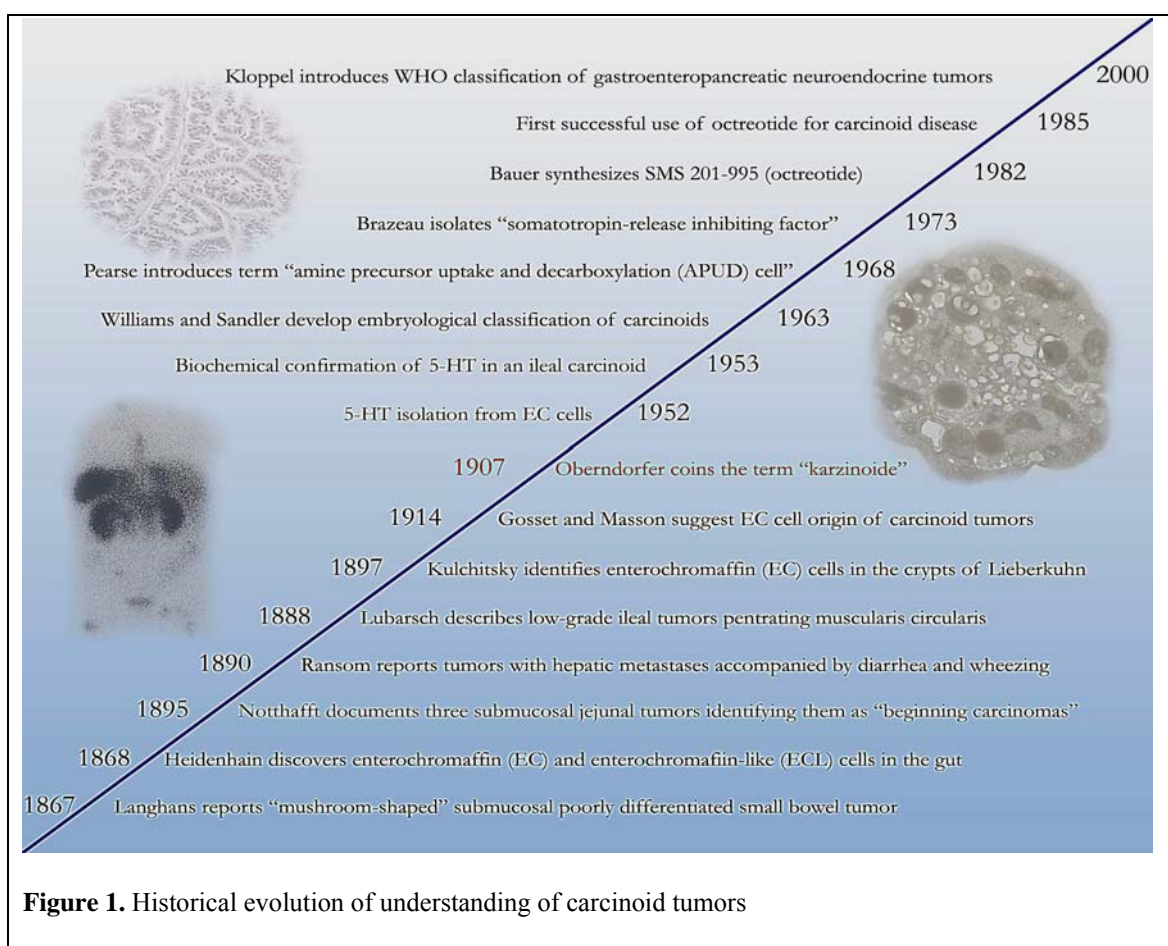
### ***1. a. Evolution of Understanding***

Carcinoid tumors are rare, frequently indolent neoplasms that, although clinically well-defined, are regarded as exotic and are consequently often unrecognized. Although little is known of the men who are credited with the initial description of its distinct histology and cell type, even less is known of the pathobiology of the lesion. Because these lesions exhibit a high degree of morphologic and biologic heterogeneity, there is a lack of clarity regarding their individual characteristics. A more generic term, NE tumor (NET) has been introduced to replace the term carcinoid and such lesions are currently referred to as gastroenteropancreatic (GEP) NETs (GEP-NETs). This notwithstanding, the classification still requires further refinement because a substantial group of NETs are of

identifiable malignant potential and represent an indistinct biologic group whose behavior cannot be accurately predicted. This reflects the fact that traditional morphologic criteria of neoplasia have limited applicability. Molecular characterization (as yet lacking) is required to refine and further differentiate GEP-NETs.<sup>5</sup>

### *1. b. Early Observations*

The earliest recorded descriptions of what were most likely carcinoid tumors as we know them today came in the second half of the nineteenth century. T. Langhans (1839-1915), O. Lubarsch (1860-1933), and W. B. Ransom (1860-1909) described unusual tumors in the small bowel but each failed to adequately investigate these novel entities [Figure 1].



**Figure 1.** Historical evolution of understanding of carcinoid tumors



In 1867, T. Langhans described a firm, mushroom-shaped submucosal tumor projecting into the lumen of the intestine without evidence of peritumoral invasion.<sup>6</sup> Histologically, the tumor resembled poorly differentiated glandular tissue arranged in ‘nests’ with a rich, thick fibrous stroma. Thereafter in 1888, Lubarsch reported the post-mortem identification of multiple ileal tumors with microscopic evidence of low-grade penetration into the muscularis circularis as well as hyperplasia of the adjacent muscularis mucosae.<sup>7</sup> Commenting upon the unique characteristics of these tumors, Lubarsch resisted classifying them as carcinomas.

Two years later, W.B. Ransom provided the first detailed descriptions of the classical symptomatology of carcinoid syndrome – wheezing and diarrhea - in a patient with an ileal carcinoid tumor and hepatic metastasis. Aside from histological similarities to carcinomas, Ransom furthermore noted that carcinoid tumors “*may remain undetected for a long time...[and] demonstrate very slight, local malignancy or tendency to infiltrate or destroy their surrounding tissues*”.<sup>8</sup> In an autopsy report of 1895, A. Notthafft documented three submucosal tumors of the upper jejunum that he described as “*beginning carcinomas*”.

### ***1. c. Karzinoide and Oberndorfer***

Despite these early descriptions, the definition of carcinoids as neoplasms distinct from carcinoma had still not been established. This responsibility fell to Siegfried Oberndorfer (1876-1944). During his tenure at the Pathological Institute of the University of Munich, Oberndorfer noted in 1907 that these lesions were distinct clinical entities and named them “karzinoide” (“carcinoma-like”), emphasizing in particular their benign features. These observations were first presented at the German Pathological Society meeting in

Dresden in September 1907 and in December of the same year, published his groundbreaking report “Carcinoid Tumors of the Small Intestine” in the *Frankfurt Journal of Pathology*.<sup>9</sup>

Oberndorfer described their distinct characteristics: “*they are mostly small and multiple, tend to be surrounded by undifferentiated tissues, have the potential to become invasive, do not metastasize, and grow extremely slowly and are, therefore, of a harmless nature*”.<sup>9</sup> The tumors consisted of small polymorphic cells with prominent nuclei and scant cytoplasm arranged in nests surrounded by dense, fibrous connective tissue composed of surrounding stroma with epithelial vascular growth adjacent to the tumor. Since the tumors appeared to have unique clinical characteristics distinguishable from those evident in carcinomas, Oberndorfer labeled them as “carcinoid-like” or “*karzinoide*”.<sup>9</sup>

Although Oberndorfer’s early contributions to the understanding of the biology of carcinoid tumors were farsighted, the characterization of these lesions as benign subsequently proved to be incorrect. Twenty-two years after first describing the tumor, Oberndorfer elaborated his further experience with thirty-six appendiceal and small intestinal carcinoids, amending the initial characterization of the benign behavior and confirming the possibility that “*karzinoide*” might exhibit malignant features and indeed metastasize.<sup>10</sup>

Although the enterochromaffin cell, the carcinoid cell of origin, had been identified as early as 1897 by N. Kulchitsky (1856-1925), it was not until 1953 that F. Lembeck (1922–) established that such cells synthesized and secreted serotonin—a potent bioactive amine. Thereafter the clinical effects of serotonin, including “flushing,”

were recognized as was the associated relationship of carcinoid heart disease (Biörck in 1952) and fibrosis (Moertel in 1961).<sup>11</sup>

The recognition of carcinoids as endocrine-related tumors was first outlined by Gosset and Masson in 1914.<sup>12</sup> In 1963, Williams and Sandler classified carcinoids according to their embryologic site of origin as foregut carcinoids (respiratory tract, stomach, duodenum, biliary system, and pancreas), midgut carcinoids (small intestine, appendix, cecum, and proximal colon), and hindgut carcinoids (distal colon and rectum).<sup>13</sup> This classification was the first to emphasize clinicopathologic differences between the tumor groups composing the gastroenteropancreatic NE tumors (GEP-NETs) but never achieved general acceptance in routine diagnostic practice because it proved too imprecise to distinguish between the different biologically relevant GEP-NET entities.

#### ***1. d. WHO Classification***

The first WHO classification of endocrine tumors (1980) applied the term *carcinoid* to most NETs, exempting the endocrine tumors of the pancreas and thyroid, paragangliomas, small-cell lung carcinomas, and Merkel cell tumors of the skin. Carcinoids were divided into enterochromaffin (EC) cells, gastrin (G) cells, and an unspecified category. This, however, has led to misunderstandings between pathologists and clinicians because the former applied the term *carcinoid* to all tumors with NE features, whereas the clinicians used the term *carcinoid* in reference to a serotonin-producing tumor exhibiting the now ubiquitously recognized carcinoid syndrome. A further issue was the growing awareness of the heterogeneity of such tumors, and it was no longer possible to equate a gastric with an ileal or rectal carcinoid.

The neoplasms of NE origin that are recognized by the most recent WHO classification (2000) consist of pure endocrine tumors, mixed endocrine-exocrine tumors and tumor-like lesions. For pure endocrine tumors, a uniform scheme of classification is applied for all anatomical sites, identifying three categories: (1) well-differentiated endocrine tumors with benign or uncertain behavior at the time of diagnosis; (2) well-differentiated endocrine carcinomas with low-grade malignant behavior, and (3) poorly differentiated endocrine carcinomas, with high-grade malignant behavior.<sup>14</sup> However, to preclude confusion, the term *carcinoid* was not completely abandoned, but is used synonymously with the term “well-differentiated NE tumor”. The term “malignant carcinoid” is used synonymously with the term *well-differentiated NE carcinoma*.

Overall, the objective of these multiple revisions was to provide a prognostically relevant classification system that assessed tumors according to size, proliferative activity, angioinvasion, organ invasion, metastases, hormone activity, and clinical syndromes in order to establish a rational basis for predicting prognosis.<sup>15</sup>

The elucidation of the pathobiology of NETs, however, is hampered by the lack of scientific tools that define their mechanisms of secretion, proliferation, and metastasis. Molecular biologic techniques and genetic analysis may facilitate the delineation of the molecular pathology of NETs and provide novel insights into their cellular mechanisms.<sup>16</sup>

The most recent assessment of the molecular basis of tumorigenesis of GEP-NETs noted multiple differences in chromosomal aberrations and gene expression patterns between gastrointestinal neuroendocrine and pancreatic endocrine tumors, with a few areas of overlap in the accumulation of genetic aberrations. These data suggest that the recent WHO classification of GEP-NETs may require updating.<sup>5</sup>

## 2. Pathology

Carcinoid tumors are *usually* classified by their embryonic gut origin, and the ubiquitous, yet inconsistently defined, classification of “typical” vs. “atypical” carcinoids has become prevalent within the literature, usually in reference to their degree of differentiation. “Typical” carcinoids, by definition, are tumors with NE differentiation and classical histologic architecture of trabecular, insular, or ribbon-like cell clusters, with no or minimal cellular pleomorphism and sparse mitoses.<sup>17</sup> “Atypical” carcinoids, however, refer to aggressive forms of poorly differentiated carcinoid tumors with increased mitotic activity and the absence or limited extent of necrosis.<sup>18</sup>

As mentioned above, the term *carcinoid* is no longer adequate to cover the entire morphologic and biologic spectrum of neoplasms of the disseminated NE cell system. In the last two decades, knowledge of the cellular origins and biologic behavior of GEP-NETs has increased greatly, due to advances in clinical and morphologic diagnostics. As a result, a more refined view of the classification and treatment of GEP-NETs has developed. This supports the need to retire the archaic concept of “carcinoid.” Classification based on embryological origin (foregut, midgut, and hindgut) is an outdated but somewhat useful distinction because the features of carcinoid tumors derived from each respective location differ clinically, histologically, and immunochemically. Thus, foregut and hindgut carcinoids are typically argentaffin negative, contrary to midgut lesions that are argentaffin positive.<sup>19</sup> More recently, sophisticated modern methods of analysis have fostered the development of precise classification systems that can discern the motley assortment of peptides and amines present in carcinoid tumors. Current estimates indicate the identification of as many as 40

different secretory products in the different varieties of carcinoid.<sup>20</sup> The diagnosis of carcinoid tumors is also supported by ultrastructural findings of intracytoplasmic electron-dense secretory granules and by immunoreactivity with antibodies to chromogranin A (*CgA*).<sup>21</sup>

### ***2. a. GEP-NET Histology***

Phenotypically, the cells of the GEP-NETs may be considered as part of the disseminated NE cell system, first referred to as “Helle Zellen” (clear cells) and subsequently defined by Pearse as “APUD cells.”<sup>22</sup> These cells are scattered throughout the GI mucosa or in the pancreas where they form aggregates as described by Langerhans.<sup>22</sup> The term “NE” reflects the phenotypic relationship to neural cells, more specifically pertaining to the expression of certain common proteins, including neuron-specific enolase (NSE), synaptophysin, and *CgA*. These proteins have utility as general markers in the morphologic diagnosis of GEP-NETs because, for the most part, they are independent of cell-specific hormone production. More specific markers of the normal and neoplastic NE cells are the bioactive products (hormones) of the GEP system. Although at least 12 different types of endocrine cells are currently recognized, less than half of the known hormones are expressed in GEP-NETs.

In addition, it is of interest that the organ in which a particular hormone producing tumor originates appears to confer biologic and clinical significance in determining outcome. Thus, duodenal gastrinomas exhibit a far less aggressive behavior pattern than pancreatic tumors derived from the same cell type (G cell).<sup>23, 24</sup>

## ***2. b. Tumor differentiation***

To facilitate more accurate delineation of malignancy of neuroendocrine lesions and hence establish more objective criteria of prognostication, the most recent WHO classification placed particular emphasis on tumor proliferative activity.<sup>25</sup>

### ***2. b. i. Well-differentiated tumors***

Well-differentiated lesions by definition widely and diffusely express all general markers of NE differentiation (CgA, synaptophysin, NSE) and may be associated with specific hormonal syndromes (carcinoid syndrome, Zollinger-Ellison).<sup>14</sup> Tumors composed of ECL cells are found only in the stomach, insulin cell tumors in the pancreas and gastrin-producing cell tumors are solely observed in tissues where normal gastrin-producing cell is present during the adult or embryonic life (antrum, upper intestine and pancreas).<sup>14</sup> EC cell tumors are observed throughout the gut and the pancreas, but are most common in the ileum and appendix. Well-differentiated tumors may be of a tentative grade 1 histology characterized by a variable structure, either with solid islet, pseudoglandular, trabecular or mixed patterns, with low atypia and rare mitoses.<sup>26</sup> Tentative grade 2 histology may be characterized by a more solid structure with focal spotty necrosis and by more moderate atypia and focal mitoses.

The latter more aggressive tumor features prompted the search for histopathological criteria of malignancy. Some of them refer to the classical tumor staging system consisting of tumor size, wall invasion or invasion of surrounding tissues, angioinvasion or perineural space invasion. Other variables pertain to the presence of atypia, mitotic index, Ki67 index, p53 overexpression and the ploidy status.

### ***2. b. ii. Poorly-differentiated tumors***

Poorly-differentiated endocrine tumors do not express specific cell types, are mostly composed of protoendocrine cells of small to intermediate size, and are not associated with hyperfunctional syndromes.<sup>14</sup> These lesions display severe grade 3 histology with a prevalent solid structure, abundant central necrosis, severe cytologic atypia with frequent atypical mitoses, high Ki67 proliferation index and frequent p53 abnormalities. Chromogranin A is normally absent or focally expressed, while synaptophysin and NSE are strongly and diffusely expressed.

### ***2. c. Appendiceal carcinoid histology***

Appendiceal epithelium is composed of colonic type mucin-secreting cells, diffuse NE cells of the crypts, and Paneth cells, which are functionally similar to neutrophils and provide host defense against microbes in the intestine.<sup>27</sup> In addition, a population of subepithelial NE cells located in the lamina propria has also been described.

The initial histologic identification of APCs came in 1928 when Masson described the subepithelial “Kultschitzky” cells as the origin of APC tumors and demonstrated that these cells exhibit both endocrine and neural characteristics.<sup>28</sup> Unlike epithelial NE cells, which give rise to carcinoids in other locations, and which are uniformly distributed along the appendix, the subepithelial NE cells are much more numerous at the tip of the organ.<sup>29</sup> A subsequent study by Shaw confirmed the neuroectodermal origin of APCs and noted the preponderance of subepithelial NE cells near the tip.<sup>30</sup> This distribution is consistent with the observation that 70%–80% of APCs occur at the tip, 5%–20% in the body, and only 7%–8% at the base of the organ.<sup>30</sup> Tumor location is of particular importance, since lesions that arise near the base of the appendix



are more likely to present symptomatically, either as obstructive appendicitis or mucocoele.<sup>31</sup> In addition, while the density of epithelial NE cells remains stable, the subepithelial NE cells vary throughout their lifespan. Their density is low in infants, increases with age to a peak around the fourth decade, and thereafter slowly declines in the elderly.<sup>14</sup>

#### ***2. d. Histologic subtypes of appendiceal carcinoids***

Although APCs are frequently considered a very homogeneous pathologic entity, their characteristics and behavior vary widely when individual histologic subtypes are considered. Appendiceal adenocarcinomas possess an identical phenotype to that of colonic tumors, whereas the “conventional” carcinoid tumors of the appendix exhibit an exclusively NE phenotype.

Appendiceal tumors exhibiting both NE differentiation and mucin production and/or glandular differentiation are rare and are regarded as “variants” of the “true” APC.<sup>32</sup> Such lesions have previously been variously designated as adenocarcinoid, GBC carcinomas (GBC), and mixed adenocarcinoma-carcinoid. In the latter instance, the distinct signet ring cell features have occasionally led to the diagnosis of poorly differentiated adenocarcinoma.<sup>4, 33</sup>

The term goblet-cell carcinoid was introduced in 1974 for tumors of the appendix exhibiting histological features which differ from both ordinary carcinoid and adenocarcinoma.<sup>34</sup> The principal cell type was described as closely resembling the normal GBC of the epithelium of the intestinal tract, with Panetta’s and argentaffin cells being present in considerable numbers. Initially these tumors were suspected of being low-grade malignant tumors exhibiting some potential for recurrence and metastases,

adequately ‘cured’ by appendectomy.<sup>34</sup>

The term ‘adenocarcinoid’ was coined in 1978, and despite abundant mucin production and goblet-cell arrangement, a closer relationship to carcinoid than to adenocarcinoma was postulated.<sup>35</sup> Size was not supposed to be a sufficient prognostic factor, but neoplasms generally confined to the muscularis propria never exhibited metastases.<sup>29</sup> Consequently, appendectomy was seen as adequate treatment of those ‘adenocarcinoids’ exhibiting no further signs of malignancy. However, in a later series five patients with diffuse appendiceal involvement all developed intra-abdominal metastases.<sup>36</sup> As a result of this experience, hemicolectomy was recommended for invasive lesions.<sup>37</sup> More extensive surgery may also be warranted for the removal of potentially positive appendiceal margins, which are generally present in the diffuse type, or to remove early lymphatic spread, which is reported in 12.5% of patients.<sup>38</sup> Recommendation for the more aggressive approach was adopted by a greater part of the surgical community, which is why approximately 45% of goblet-cell carcinoids were treated by right hemicolectomy.<sup>29</sup> In accordance to the 2002 guidelines of the German Cancer Society, GBC tumors are now categorized as low-malignant carcinomas of the appendix.<sup>29</sup>

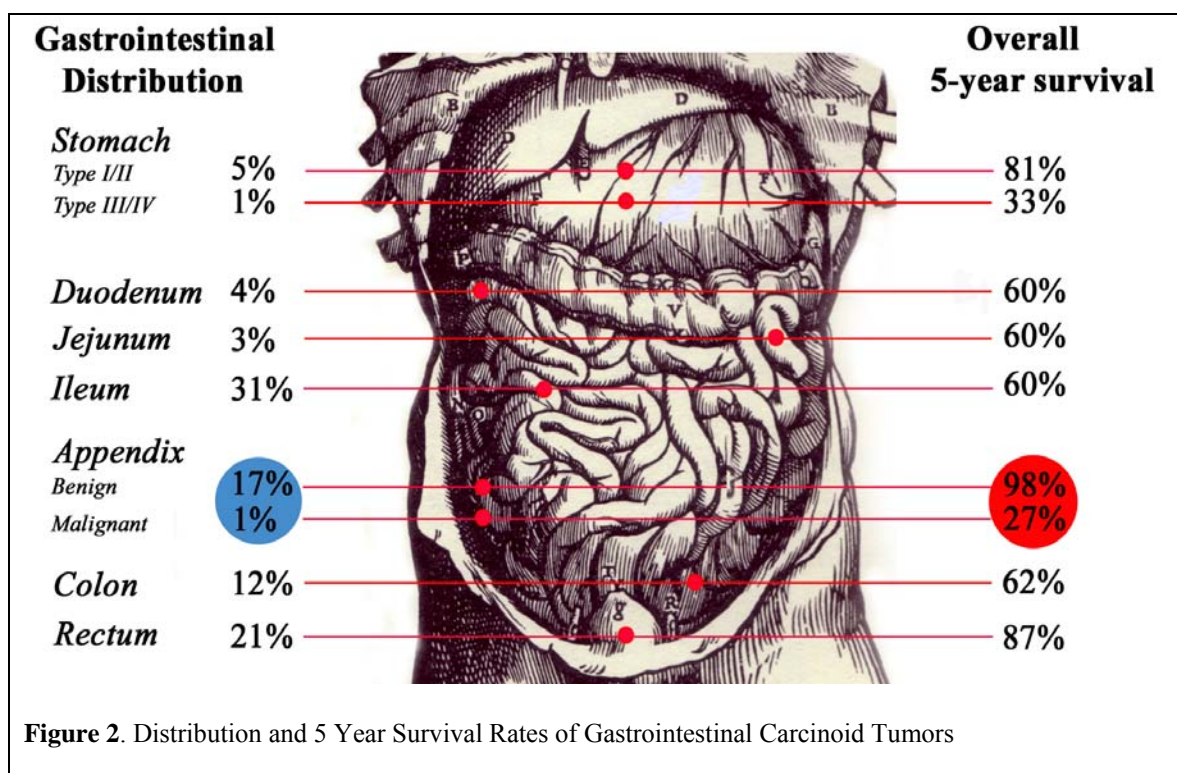
It remains a matter of controversy whether GBCs should be considered adenocarcinomas or as part of the carcinoid tumor spectrum.<sup>16</sup> They appear to arise from subepithelial lamina propria without association with intraepithelial NE cell hyperplasia or dysplasia of the appendiceal crypt epithelium. Because both the clinical and the pathologic features of the GBC carcinoid are sufficiently distinctive, they are probably best recognized as a separate entity.

### ***3. Epidemiology***

Approximately 75% of carcinoid tumors occur in the GI tract, the most common sites being the small bowel (38%), appendix (18%) and rectum (21%). They can either be sporadic or occur as part of familial syndromes (0-85%) such as multiple endocrine neoplasia I and II (MEN), Von Hippel Lindau syndrome (VHL) and neurofibromatosis (NF).<sup>39, 40</sup>

APCs are the most frequent neoplasms of the appendix, comprising 32–57% of all appendiceal tumors, although this fraction seems to have decreased over time from 31.8% in the period 1973–1979 to only 12% in 1990–1997.<sup>2, 41, 42</sup> These usually small, apparently benign lesions are often discovered as an incidental finding during surgery. The great majority are no larger than a pea, and it is extremely rare for them to exceed the diameter of a quarter.<sup>31</sup> Identification of the lesion occurs in 5 or 6 per 1000 appendectomies but an exact incidence is unknown because many lesions remain asymptomatic.<sup>42</sup> An autopsy series of 16,294 cases between 1958 and 1969 identified APCs in 0.04% of individuals.<sup>43</sup> This translates to prevalence rates of 8.4 per 100 000 population per year.<sup>43</sup> The comprehensive analysis of the Surveillance, Epidemiology and End Results program of the National Cancer Institute (SEER), however, recorded 4.48 cases per 100 000 population per year.<sup>29</sup> This possibly implies that a considerable percentage of carcinoid lesions remain asymptomatic and undetected throughout the life span of a patient. Reported epidemiology may therefore reflect more a change in diagnostic and therapeutic behavior than a real change in prevalence of the disease itself.<sup>29</sup> The true number is assumed to be much higher because the use of immunocytochemistry in the detection of NE tumors is a relatively recent development.

Though previously recognized as the most frequently occurring of carcinoid tumors, the relative frequency of appendiceal tumors appears to have decreased over time (4.7% of all carcinoid tumors and 18% of all GI carcinoids [Figure 2].<sup>44</sup>



Possible contribution to this relative decrease in incidence has been the widespread use of endoscopy, ultrasonography, computerized tomography, MRI, and somatostatin receptor scintigraphy (OctreoScan), which have significantly enhanced the identification of previously undetectable lesions in locations that are not explored as routinely and readily as the appendix.<sup>45</sup> As a consequence, carcinoid tumors of the gut “appear” to have increased in incidence over the last 20 years.<sup>44</sup> Additionally, the overall incidence of primary appendectomy decreased by approximately 20%, with consequently fewer APCs being detected.<sup>45</sup>

The persisting impression that the appendix is the most common site might have arisen from the fact that removal of the appendix is one of the most frequently performed

operations, as there is almost no other organ in the human body which is more often available for histopathological examination, either following surgical removal for signs and symptoms of acute appendicitis or following exploration during pelvic procedures performed in women, which is probably even more extended by the introduction of laparoscopy facilitating this type of invasive diagnostic intervention.<sup>44,29</sup> Consequently, several studies reported a preference for female gender (over 80%).<sup>38, 46</sup> Although this pattern has decreased from 77% to 57% in the latest SEER data analysis.<sup>44, 47</sup>

In addition, APCs present in a younger patient population than other GI carcinoid tumors, with a median age of 49.3 years, which probably reflects the role of appendectomy in the identification of such lesions.<sup>44</sup> When they are found in the elderly, they are frequently calcified, with only a few remnant tumor cells.<sup>31</sup> Of note, those with larger tumors and metastases are usually younger (29 years of age) than those with smaller and clinically “benign” lesions (42 years of age).<sup>48</sup>

#### 4. *Clinical Features*

As was already suggested, many APCs are asymptomatic, found incidentally and diagnosed only by post surgery histopathological examination [**Table 1**].

	Stomach	Small bowel	Appendix	Colon	Rectum
Carcinoid syndrome	1+	2+	1+	1+	1+
Weight loss	1+	1+	1+	3+	2+
Vomiting	2+	1+	1+	1+	1+
UGI bleeding	1+	2+	1+	1+	1+
Rectal bleeding	1+	1+	1+	2+	2+
Obstruction	1+	3+	1+	1+	1+
Constipation	1+	1+	1+	1+	1+
Palpable mass	1+	2+	1+	2+	1+
Pain/discomfort	2+	2+	2+	3+	2+
Asymptomatic	2+	2+	3+	1+	2+

**Table 1.** Frequency of symptoms in gastrointestinal carcinoids

Abbreviations: aqua 1+: rare (<10%); yellow 2+: modest (11-50%); red 3+: frequent (>50%)

Nonspecific abdominal pain in the lower right abdomen frequently leads to appendectomy, with some broadening of the appendix tip detected on surgery.<sup>4</sup> More aggressive goblet-cell carcinoids often present with a diffusely inflamed appendix.<sup>29</sup> Although some radiologists recommend CT scanning whenever an appendiceal mass is suspected, routine application of this technique is not likely to really increase the preoperative diagnosis of this rare entity. Additionally, improvement in preoperative imaging is unlikely due to the similarity of NETs to inflammation, and therapeutic decision-making would not be altered. The same is true for ultrasound, which because of its universal availability and non-invasiveness has become a useful tool in the diagnosis of appendicitis. However, the differential diagnosis of a small (<1 cm) APC compared to a local inflammation process is still a very distant hope.

Systemic manifestations of carcinoid disease include flushing, sweating, diarrhea, or bronchospasm, are often paroxysmal and are the result of the secretion of bioactive substances by either the primary lesion or metastases. However, carcinoid syndrome is rare and requires abundant disease or liver metastases.<sup>49,50</sup> In addition, cardiac failure may occur as a result of tricuspid or pulmonary valvular fibrosis.

As for other gastrointestinal NETs, a significant number (7 to 48%) of coexistent malignant tumors can be found, primarily throughout the gastrointestinal tract, which is the reason why patients with proven appendiceal NET should undergo total colonoscopy and have a complete diagnostic workup.<sup>29</sup> Of note is the yet to be elucidated association of APCs with Crohn's disease. Small appendiceal NETs (<1 cm) have an excellent prognosis after appendectomy specimens of Crohn's patients revealed an incidence of 0.3

carcinoids, exceeding the reported rates of detection of these lesions after removal of the appendix for appendicitis or the rate of APCs in autopsy studies.<sup>51</sup>

## ***5. Clinical relevance***

Keeping in mind that carcinoid may be the final pathological diagnosis in approximately one in 300 patients undergoing appendectomy, every surgeon has a considerable probability of facing this tumor type.<sup>48</sup> Realizing an average appendectomy rate of 100 operations per year in a community hospital, APC remains a persistent problem featuring approximately one or two cases a year per hospital. Although APCs have the best prognosis among all types of carcinoids [**Figure 1**], this primarily reflects the anatomic location and easy availability for exploration, which leads to early detection and removal.

### ***5. a. Classic determinants of malignancy***

#### ***5. a. i. Tumor size***

As for most tumors, risk of metastases is associated with tumor size. Although early reports indicate that 4.6% of APC present metastatically on diagnosis, this number is much less than the 38.8% of the late SEER series.<sup>44, 48</sup> However, the criteria for NET registration in this surveillance program changed over time. Namely, while the early series collected data on miscellaneous ‘carcinoid’ tumors of the appendix, the late registration collected data only on NETs specifically classified as ‘malignant carcinoids’.<sup>38</sup>

The risk of metastases in lesions <1 cm is virtually zero.<sup>29</sup> The tumors between 1 and 2 cm are metastasized in 0–1%. Lesions larger than 2 cm, however, are non-localized in 20–85%.<sup>47, 52, 53, 48, 54</sup> Furthermore, 60–76% of APCs are smaller than 1 cm, 4–27% are

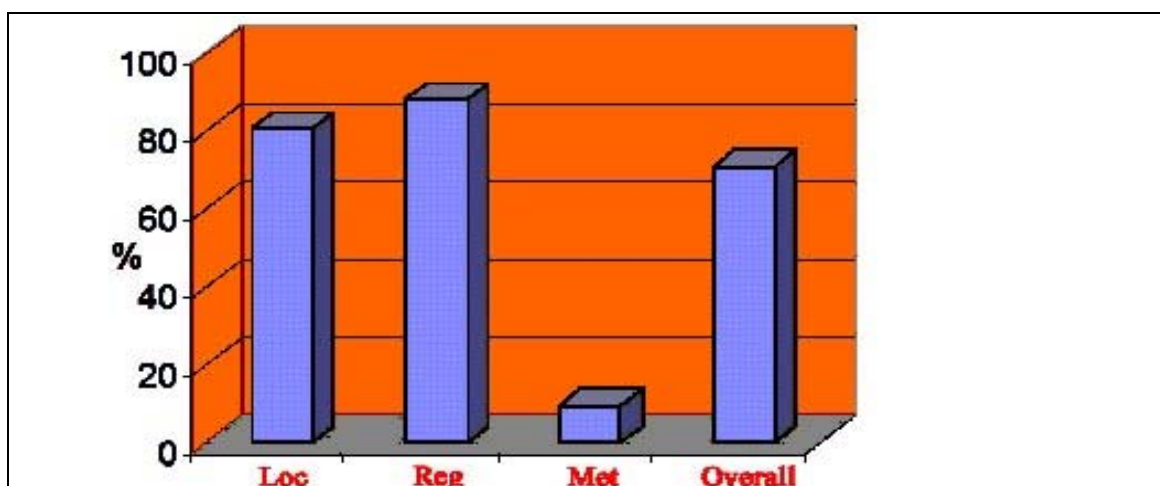
1–2 cm, and 2–17% are larger than 2 cm in diameter.<sup>55</sup> In the Mayo Clinic series 21% of APCs 2–3 cm and 44% of lesions >3 cm were metastasized at diagnosis, but no tumor measuring <2 cm exhibited metastatic spread.<sup>48</sup>

#### *5. a. ii. Tumor invasion*

In addition to tumor size, the metastatic potential depends greatly on the depth of penetration and the site of origin.<sup>56</sup> Thus, mesoappendiceal invasion occurs more frequently in patients with distant and lymph node metastases and should be used as a determinant in indicating the need for right hemicolectomy.<sup>57</sup> Some reports, however, have suggested that the invasion of the mesoappendix is not a reliable predictor of metastatic potential.<sup>54, 58</sup> Serosal involvement is demonstrated to be present in about 70% of all malignant NETs, but is deemed to be unrelated to outcome in the published literature.<sup>29</sup> Contradictory data are given for vascular invasion, which in some studies is not significantly related to outcome, whereas it is regarded as a feature of elevated malignant potential in others.<sup>4</sup>

Five-year survival rates for localized lesions, regional spread, and distant metastases are 80.8%, 88.1%, and 9.6%, respectively, with an overall survival rate of 71% [**Figure 3**].<sup>44</sup>





**Figure 3.** Five Year Survival.

Loc – lesion described as in situ or confined to organ of origin;

Reg – local invasion or lymph node metastasis;

Met – evidence of metastatic invasion of other organs;

The overall 5-year survival rate is among the best of all types of carcinoids and reflects both the early and often serendipitous detection of the tumor as well as, in most cases, the modest biological behavior of the lesions.<sup>4, 33, 44</sup> These data do not, however, differentiate tumors into specific subtypes such as high- or low-grade GBC. For instance, the survival of patients with mucinous variants is far less propitious and is estimated overall to be  $\sim 26 \pm 19$  months.<sup>59</sup>

### ***5. a. iii. Tumor histology***

To optimize phenotypic assessment and optimize therapeutics of gastrointestinal NE tumors, a comprehensive classification has been proposed [Table 2].<sup>14</sup>

Biological behavior	Metastases	Invasion of muscularis propria	Histological differentiation	Tumor size, cm	Angioinvasion	Ki-67 index, %	Hormonal syndrome
Benign	-	-	WD	≤1	-	<2	-
Benign or low-grade malignant	-	-	WD	≤2	-/+	<2	-
Low-grade malignant	+	+*	WD	>2	+	>2	+
High-grade malignant	+	+	PD	any	+	>30	-

**Table 2.** Criteria for prognosis assessment of gastrointestinal NETs.

\*Exception: benign NETs of the appendix usually invade the muscularis propria; WD – well-differentiated; PD – poorly differentiated

In addition to physical properties of individual lesions (size, invasion), the presently available tools for tumor evaluation also rely on the antigenic asset of NE cells and the tumor classification criteria.<sup>14</sup> The former comprises molecules of the endocrine cell machinery found in the cytosol (NSE) or in the secretory vesicles (CgA in large dense core vesicles and synaptophysin in synaptic-like microvesicles). These antigens are common to all NE cell types, while the hormonal content of secretory granules identifies a specific cell type. The latter consist of morphologic/biologic criteria including tumor size, angio-invasion, proliferative activity, histologic differentiation, metastases, invasion, and hormonal activity (association with clinical syndromes or diseases). Since their highly aggressive cancer-like course, the neoplasms classified by the WHO as poorly differentiated endocrine carcinomas, with high-grade malignant behavior, are profoundly different as compared to well-differentiated lesions and require an aggressive therapeutic approach.<sup>14</sup>

A combination of these findings has given shape to present surgical management

guidelines of APCs. Typically, lesions >2 cm in diameter are seen to benefit from an oncological resection of the right hemicolon. Small appendiceal NETs (<1 cm) have an excellent prognosis after appendectomy. Although the decision may be clear-cut for APCs <1 and >2 cm, the intermediate type has a small but detectable risk of metastasis and needs further evaluation. In principle, hemicolectomy is suggested in APCs 1–2 cm in size if the mesoappendix and angioinvasion are demonstrable, a high proliferative index and Ki67 level is apparent, and lesions are located at the base of the appendix with positive margins.<sup>29</sup>

Despite numerous contributions to the elucidation of the histologic, pathologic, and biochemical characteristics of GEP-NETs, including APCs, currently, there is no reliable means of predicting the malignancy and metastatic potential of these tumors.<sup>5</sup> Thus, it is apparent that the delineation of the molecular basis of such tumors is required to define tumor characterization, facilitate diagnosis, rationalize therapy, and establish prognosis.

#### ***5. b. Biological Determinants of Malignancy***

To date, optimal surgical strategies for APC tumors have been inferred from the retrospective analysis of surgical and pathological series and are based on a variety of criteria including, but not limited to - tumor size, mitotic index, meso-appendiceal invasion, lymph node spread, and location of the lesion.<sup>4, 60, 61</sup> Nevertheless, recurrences of these tumors or pseudomyxoma peritonei occur. The failure to accurately define the biology of the tumor or precisely predict its pathological behavior play a major role in these developments.<sup>4, 60, 61</sup> In the future, it is likely that the definition of specific molecular signatures will enable prediction of behavior, irrespective of size.

Pathologic staging or grading of carcinoids includes angioinvasion and differentiation is limited, whereas sentinel lymph node mapping is not routinely undertaken.<sup>25</sup> The utility of Ki-67 as a prognostic index in NE tumors has been documented, but this marker provides scant predictive information.<sup>62-64</sup> Overall, therefore, determinants of malignancy and metastasis are lacking, and what is known reflects the quantification of disease extent, as opposed to delineation of the biological behavior of the tumor. At present, no technique exists to identify whether or when a tumor is malignant and to determine whether it has already developed, or is likely to develop, metastases. Novel strategies for the identification of when a primary tumor becomes metastatic are based on molecular profiling of the tumor. In the gastrointestinal tract, methods including gene expression analyses and molecular analysis of specific genes (e.g., p53) have been undertaken in colorectal tumors.<sup>65, 66</sup>

The relationship between the size of a tumor and its malignancy is often coincidental, since metastasis occurs as a result of a series of well-characterized alterations in a variety of genes that define cell adhesion, proteolysis, migration, and angiogenesis. These regulatory genes can be identified at a molecular level and may provide the basis for generating a molecular profile of individual tumors that can be then used to predict behavior and thus allow for a refinement of therapeutic strategy.<sup>67</sup>

Histological analysis *per se* (of NE lesions especially) cannot determine if a tumor is benign or malignant, and despite considerable progress in molecular biology, molecular staging has yet to be integrated into current prognostic/predictive pathological protocols.<sup>68</sup> In the absence of this combinatorial synergistic approach, the biological basis of APC malignancy and metastasis is unknown, unpredictable and hence it is currently

not possible to accurately or adequately define appropriate surgical management. This is reflected in the large SEER (NCI) database study that concluded that the most important predictor of survival was “extent” of disease and not histology.<sup>38</sup>

### ***5. c. Experience with gastric and small intestinal carcinoids***

Small intestinal enterochromaffin cell tumors (carcinoids) and APCs are derived from the EC cell, although overall the former behave more aggressively than appendiceal lesions.<sup>33</sup> Nevertheless, both can exhibit local spread, lymph node metastasis and distant (liver) metastases, while the appendiceal GBC variants may produce myxoma peritonei and ovarian implantation lesions.<sup>69, 70</sup>

At this time, no molecular signature exists to differentiate between a malignant and benign carcinoid of the appendix. Such information would be of considerable clinical importance when appendiceal tumors are identified and the need for further surgery is uncertain, given that current management strategies are based on relatively simplistic macroscopic criteria and light microscopy.<sup>71</sup>

Our group has identified the following candidate genes to be differentially over-expressed in a variety of gastrointestinal NE cells, including EC and ECL cells: Chromogranin A (*CgA*), the mitotic regulatory gene, Nucleosome Assembly Protein 1-Like 1 (*NAPILI*), the adhesion gene, Melanoma Antigen D2 (*MAGE-D2*), the malignancy marker gene, Metastasis-Associated Protein 1 (*MTA1*) and the caspase-3 activating apoptosis gene, NAcHt Leucine-rich-repeat Protein 1 (*NALPI*).<sup>72</sup>

*NAPILI* has been demonstrated to be upregulated in hepatoblastomas compared with nondiseased adult livers.<sup>73</sup> *MAGE-D2* has been identified as a molecular marker predictive of colorectal liver metastases overexpressed in >75% of primary tumors with

metastases.<sup>74</sup> Overexpression of *MTAI* mRNA and protein has been associated with tumor invasion and metastasis in a variety of tumors, including breast, hepatocellular, esophageal, gastric, and colorectal adenocarcinomas.<sup>75-79</sup>

In previous studies we have demonstrated that the differential expression of these genes enables the delineation of localized non-metastatic (Type I/II) gastric carcinoids from aggressive sporadic or NE carcinoma type tumors (Type III/IV).<sup>80</sup> In addition, by QRT-PCR, *NAPILI* was found to be significantly overexpressed in small intestinal carcinoids compared with colorectal carcinomas and healthy tissue.<sup>81</sup> Increased levels were identified in both liver and lymph node metastases. Levels in colorectal carcinomas were the same as in healthy mucosa. *MAGE-D2* and *MTAI* were increased in primary tumors and metastases and overexpressed in carcinomas. Automated quantitative analysis demonstrated the highest levels of *MTAI* immunostaining in malignant primary small intestinal carcinoids and in metastases to the liver and lymph nodes, which were significantly increased compared with nonmetastatic primary tumors.

## ***6. Hypothesis***

**Based upon the differential expression of these genes in gastric and small intestinal carcinoid tumors, we hypothesize that these genes will enable discrimination between different types of appendiceal tumors (non-malignant and those identified incidentally at surgery during routine or acute appendectomy versus aggressive and metastatic).**

## 7. Methodology

To establish and verify the clinical utility of a PCR-based protocol for the tissue resource, we initially examined archival material. For this, we utilized paraffin-embedded tissue and archival samples which constitute the majority of bankable tissue available for analysis. We examined archival paraffin-embedded samples (collected between 1965-2004 by the Yale Department of Pathology) to evaluate the expression of the marker genes of interest and correlated their expression with clinical data, tumor size and the presence of clinically and histologically documented metastasis. Thereafter, we prospectively examined gene expression in surgically collected appendiceal samples, largely from patients with acute appendicitis, to establish the utility of this molecular approach in readily available samples. These studies were approved by the Human Investigations Committee at Yale University School of Medicine (HIC # 12589).

### 7. a. Tissue specimens:

Paraffin-embedded tumor tissue blocks were collected from twenty-five patients (M:F = 8:17; median age [range] = 40 yr [11-95]) with histologically-proven APC tumors who had undergone surgical resection for acute appendicitis or a primary tumor between 1965 and 2004 in the Yale University Department of Surgery. Control tissue included colorectal adenocarcinomas ( $n=5$ ) and normal tissue samples from adjacent, macroscopically normal, non-tumor mucosa ( $n=5$ ) were also examined.

Appendiceal samples were prospectively collected from twelve patients (M:F = 8:4; median age [range] = 19 yr [6-38]) with acute or suppurative appendicitis ( $n=11$ ) and one histologically-proven invasive APC tumor [including mucosa ( $n=2$ ); omental

( $n=3$ ) and liver metastases ( $n=1$ )] who had undergone emergency surgical resection in 2004 at the Yale University Department of Surgery.

### **7. b. Tissue techniques:**

*RNA Isolation:* Paraffin blocks were deparaffinized and digested as previously described.<sup>82, 83</sup> Total RNA was isolated from paraffin-blocks or frozen sections using TRIzol reagent (Invitrogen, Carlsbad, CA) as described.<sup>84</sup> RNA was then dissolved in DEPC water, measured spectrophotometrically and an aliquot analyzed on a denaturing gel using electrophoresis to check the quality of RNA isolated.

*Q RT-PCR:* Forty-two samples were examined by quantitative real-time PCR using the Assays-on-Demand approach (Applied Biosystems) since this system identifies RNA of 60-150 base pairs in length and is thus particularly suitable for paraffin-tissue examination.<sup>83</sup> Message from *CgA*, *NAPILI*, *MAGE-D2*, *MTA1*, *NALP1* and the house-keeping gene, *GAPDH*, were quantitatively measured.<sup>84</sup> Q RT-PCR was performed using the ABI 7900 Sequence Detection System. Total RNA from each sample was reverse transcribed using a High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA) following the manufacturers instructions. Quantitative real time PCR analysis was then performed in triplicate. cDNA in 7.2  $\mu$ l of water was mixed with 0.8  $\mu$ l of 20 x Assays-on-Demand primer (*CgA* = Hs00174938; *NAPILI* = Hs00748775, *MAGE-D2* = Hs00374760, *MTA1* = Hs00183042, *NALP1* = Hs00248187, *GAPDH* = Hs99999905) and probe mix, 8  $\mu$ l of 2x TAQMAN Universal Master mix in a 384 well optical reaction plate. The following PCR conditions were used: 50°C for 2 min, then 95°C for 10 min, followed by 40 cycles at 95°C/0.15 min and 60°C /1 min. A standard curve was generated for each gene using cDNA obtained by pooling equal amounts from each



sample. The expression level of target genes was normalized to internal *GAPDH*. Data was analyzed using Microsoft Excel and calculated using the relative standard curve method (ABI, User Bulletin #2).

### ***7. c. Immunostaining of appendicitis specimens***

Triple-color immuno-staining was performed on tissue sections using monoclonal antibodies against CgA to identify the cellular location of this marker.<sup>84, 85</sup> For antigen retrieval purpose, sections were initially immersed in citrate buffer (10 mm sodium citrate, pH 6.0) and subjected to 1 x 10 min high temperature-high pressure treatment followed by treatment with 0.3% H<sub>2</sub>O<sub>2</sub> in methanol for 30 min at 37°C to inactivate endogenous peroxidase. Slides were then incubated for 24 hr at 4°C with a 1:1000 dilution of the anti-CgA mouse monoclonal antibody (DAKO Corp, Carpinteria, CA) and rabbit anti-cytokeratin antibody cocktail (AE1/AE3; DAKO Corp) (to identify tumor carcinoid cells). Goat anti-mouse antibodies conjugated to a horseradish peroxidase-decorated dextran polymer backbone (Envision; DAKO Corp, Carpinteria, CA) were used as a secondary reagent for CgA, and goat anti-rabbit antibodies conjugated to Alexa-488 fluor (DAKO Corp) were used to identify cytokeratin. CgA staining was visualized with a fluorescent chromogen (Cy-5-tyramide; NEN Life Science Products, Boston, MA) and nuclei were visualized by 4', 6-diamidino-2-phenylindole (DAPI). A pathologist (Dr. Robert Camp, Yale Department of Pathology) examined staining expression.

### ***7. d. Statistical analysis***

Results are expressed as mean  $\pm$  SEM; *n* indicates the numbers of patients in each study group. Statistical significance was calculated by the two-tailed Student's test for paired and unpaired values as appropriate, with a probability of  $< 0.05$  representing significance.

Linear regression analysis was performed to evaluate the relationship between CgA levels and tumor size.

## 8. Results

### 8. a. Clinical results

Sixteen of the 25 paraffin-embedded appendiceal tumors were carcinoids identified incidentally post-operatively with no evidence of serosal invasion or lymph node metastasis [Table 3].

Group	No.	Age [years]*	Gender [M:F]	Tumor size [cm]	Presence of Metastases	Follow-up [months]*	Subsequent Pathology
Incidental	16	30 (11-73)	6:10	0.7±0.08	None	33 (8-468)	None
Malignant	9	59 (39-95) <sup>†</sup>	2:7	2.7±0.4	LI: <i>n</i> = 3 LNM: <i>n</i> = 5 LVM: <i>n</i> = 1	113 (29-443)	<i>n</i> = 1

**Table 3.** Clinical evaluation of 25 patients with APCs

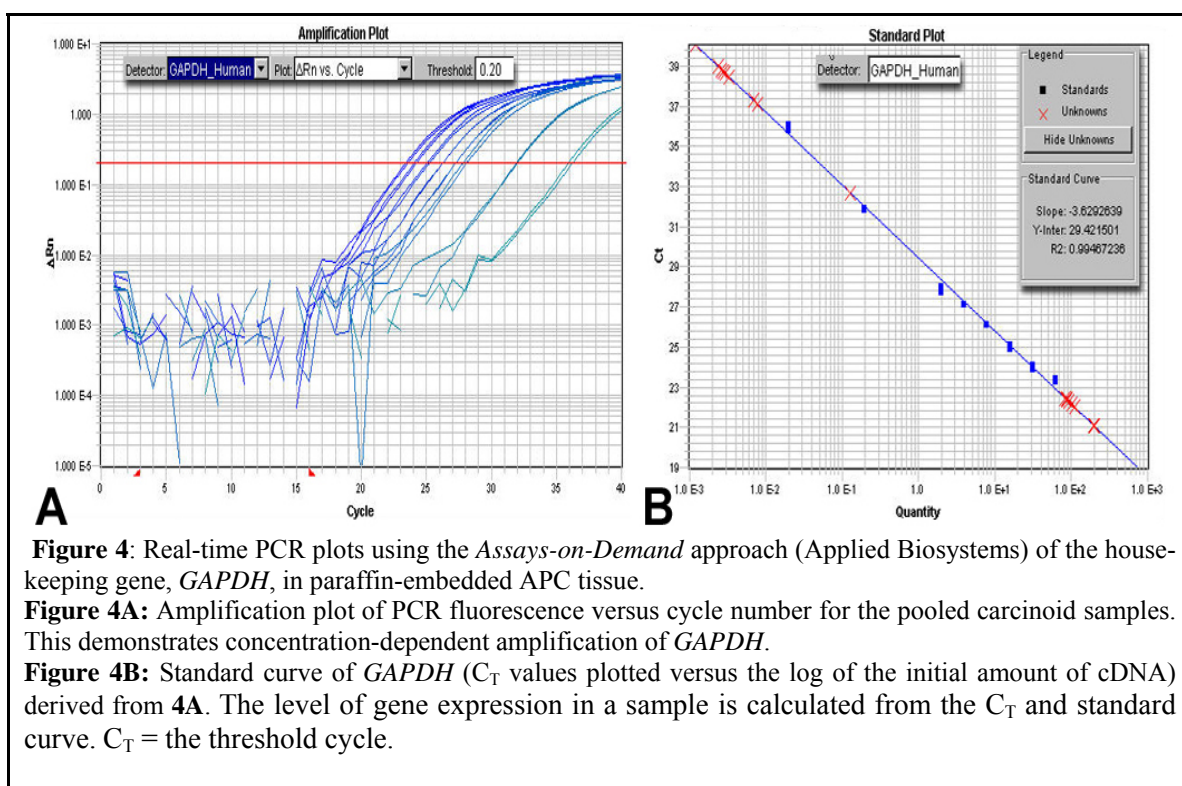
\*median values and range; LI = locally invasive, LNM = lymph node metastases, LVM = liver metastases; <sup>†</sup> *p* = 0.053 vs. “incidental”.

The mean size ( $\pm$ SEM) of these tumors was 0.68cm  $\pm$ 0.075. The mean age of the patients at diagnosis was 36.9 years and the follow-up was 113 months. None of the patients subsequently developed lymph node or liver metastases and were considered disease-free. Nine of the remaining tumors presented with local invasion and liver or lymph node metastases. Three exhibited a goblet-cell phenotype and were considered to be appendiceal adenocarcinoids. The mean size of the nine tumors was significantly greater than the sixteen incidentally-identified lesions (2.7cm $\pm$ 0.4 versus 0.7 $\pm$ 0.08; *p*<0.00002). The mean age of these patients at diagnosis was 57 years and the follow-up was 199 months. One patient subsequently developed liver metastases. All patients in this group were considered disease-specific.

The eleven fresh frozen samples had suppurative appendicitis ( $n=3$  samples with peri-appendicitis) with no pathological evidence of carcinoid tumor.

### 8. b. RNA isolation

RNA isolated from twenty-five paraffin-embedded APC tumor specimens and ten control samples had concentrations ranging from 0.02-0.14  $\mu\text{g}/\mu\text{l}$ . Using Assays-on-Demand (Applied Biosystems), *GAPDH* was amplified in all samples using Q RT-PCR [Figure 4]. These results confirm, as previously determined, that this approach is suitable for paraffin-tissue examination.<sup>83</sup>



**Figure 4:** Real-time PCR plots using the *Assays-on-Demand* approach (Applied Biosystems) of the house-keeping gene, *GAPDH*, in paraffin-embedded APC tissue.

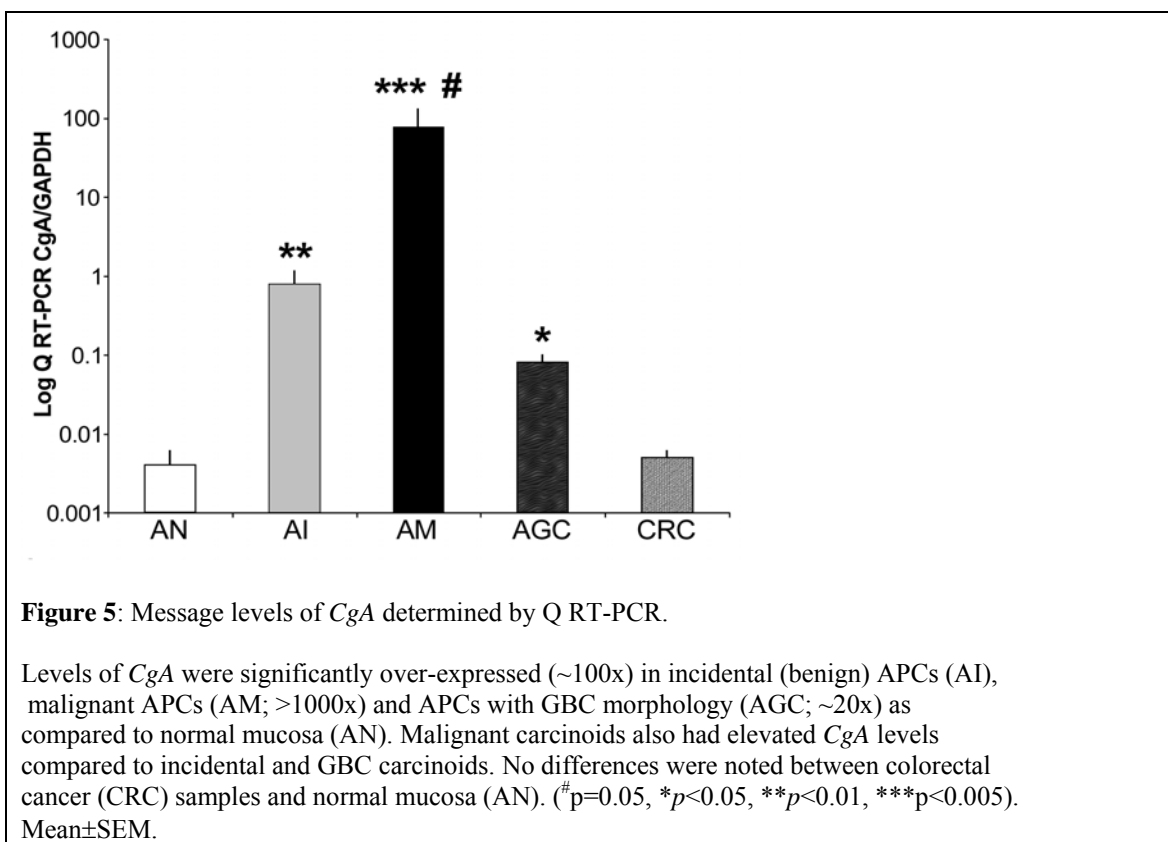
**Figure 4A:** Amplification plot of PCR fluorescence versus cycle number for the pooled carcinoid samples. This demonstrates concentration-dependent amplification of *GAPDH*.

**Figure 4B:** Standard curve of *GAPDH* ( $C_T$  values plotted versus the log of the initial amount of cDNA) derived from 4A. The level of gene expression in a sample is calculated from the  $C_T$  and standard curve.  $C_T$  = the threshold cycle.

### 8. c. Quantitative real-time PCR

#### 8. c. i. Chromogranin A:

*Chromogranin A* was amplified in all appendiceal tumor samples and was significantly elevated (100→1000-fold;  $p<0.05$ ) in the incidental and malignant appendices and ~50-fold in the GBC adenocarcinoids compared to normal mucosa and to colorectal adenocarcinomas [Figure 5].

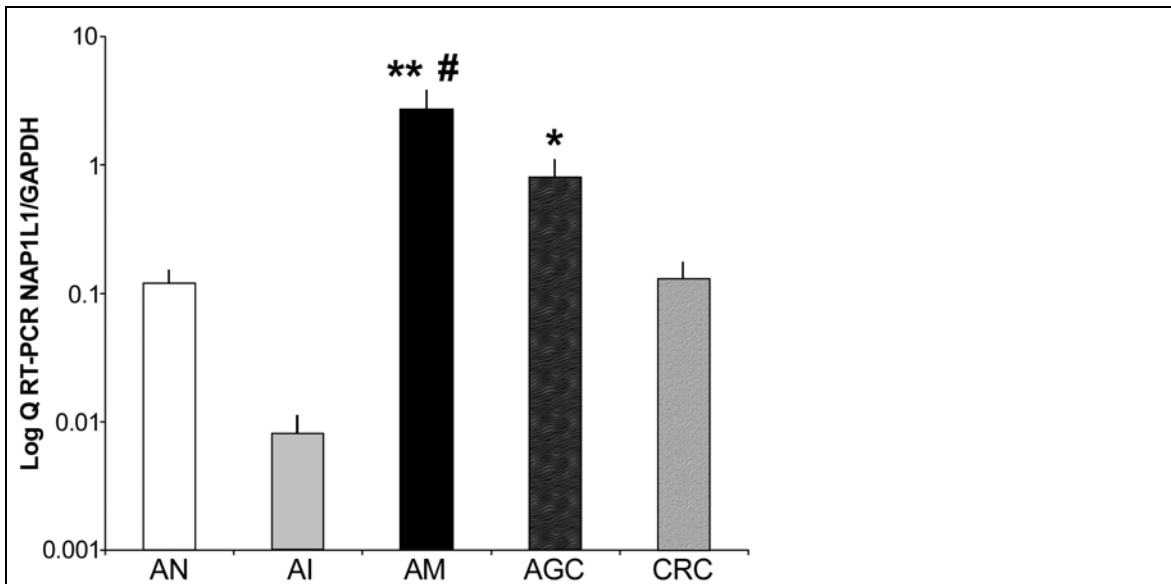


Malignant tumors also had elevated *CgA* levels compared to incidental lesions (98±41 vs. 1.02±0.6,  $p=0.048$ ). The fact that the *CgA* levels of the GBC adenocarcinoids were comparable to the serendipitously-identified lesions might be considered to reflect NE cell number. An examination of the relationship between tumor size and *CgA* message levels, however, only identified a moderate correlation between these two

parameters ( $R^2=0.304$ ,  $p=0.063$ ). Although it has been suggested that a relationship exists between plasma *CgA* and tumor size, our data suggests that tumor size and mRNA levels may not be as closely correlated as previously considered.<sup>86</sup> This is consistent with other reports indicating that cellular secretory product levels may have little relationship to plasma values.<sup>87</sup>

#### 8. c. ii. *NAPILI*:

*NAPILI* is a nuclear protein involved in chromatin assembly and DNA replication.<sup>88</sup> Messenger RNA levels of *NAPILI* were elevated >10-fold ( $p<0.03$ ) in malignant APC tumors and in GBC adenocarcinoids compared to normal mucosa. Levels were also elevated >100-fold ( $p<0.006$ ) in malignant carcinoids compared with the incidentally identified lesions [Figure 6]. Levels in colorectal adenocarcinomas were not different to normal mucosa.

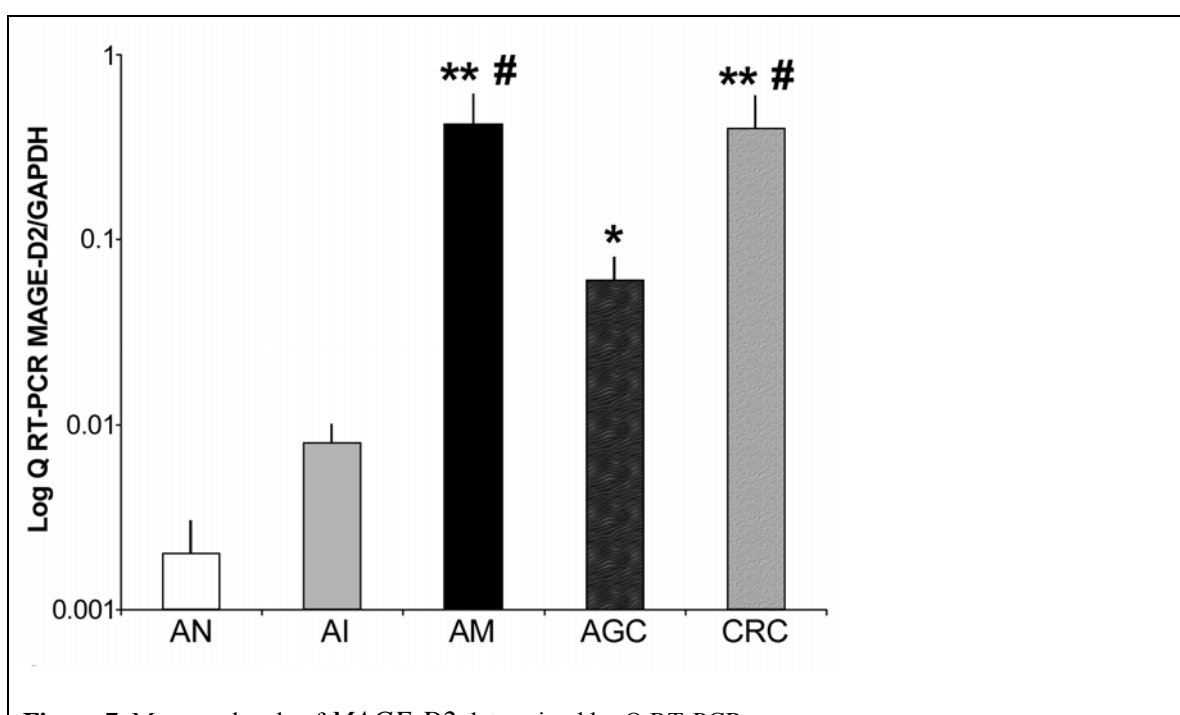


**Figure 6:** Message levels of *NAPILI* determined by Q RT-PCR.

Levels of *NAPILI* were significantly over-expressed in malignant APCs (AM; ~15x), and in APCs with GBC morphology (AGC; ~8x) compared to normal mucosa (AN). Malignant carcinoids also had elevated *NAPILI* levels compared to incidentally identified carcinoids (AI). (\* $p=0.03$ , \*\* $p<0.01$ , # $p=0.006$ ). Mean±SEM.

### 8. c. iii. *MAGE-D2*:

*MAGE-D2* is an adhesion gene and potential predictive marker of colorectal liver metastases.<sup>74</sup> Levels of *MAGE-D2* were elevated 10–100-fold ( $p<0.01$ ) in the malignant APCs, GBC adenocarcinoids and colorectal adenocarcinomas compared to normal mucosa [Figure 7]. Both malignant appendiceal tumors and colorectal tumors had elevated expression levels of *MAGE-D2* compared to incidentally identified carcinoids. No differences in expression were noted between the latter and normal mucosa.

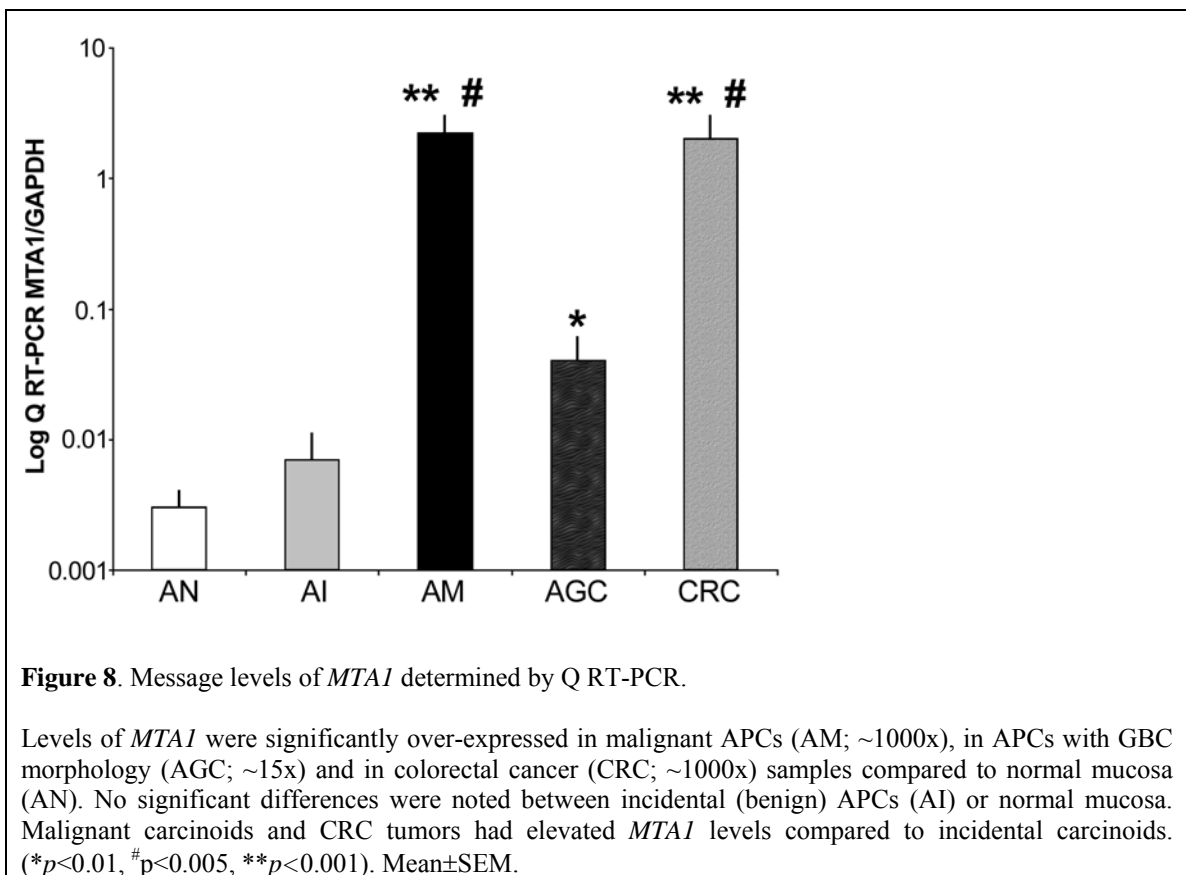


**Figure 7.** Message levels of *MAGE-D2* determined by Q RT-PCR.

Levels of *MAGE-D2* were significantly over-expressed in malignant APCs (AM; ~100x), in APCs with GBC morphology (AGC; ~12x) and in colorectal cancer (CRC; ~100x) samples compared to normal mucosa (AN). No significant differences were noted between incidental (benign) APCs (AI) or normal mucosa. Malignant carcinoids and CRC tumors had elevated *MAGE-D2* levels compared to incidental carcinoids. (\* $p<0.01$ , # $p<0.005$ , \*\* $p<0.001$ ). Mean $\pm$ SEM.

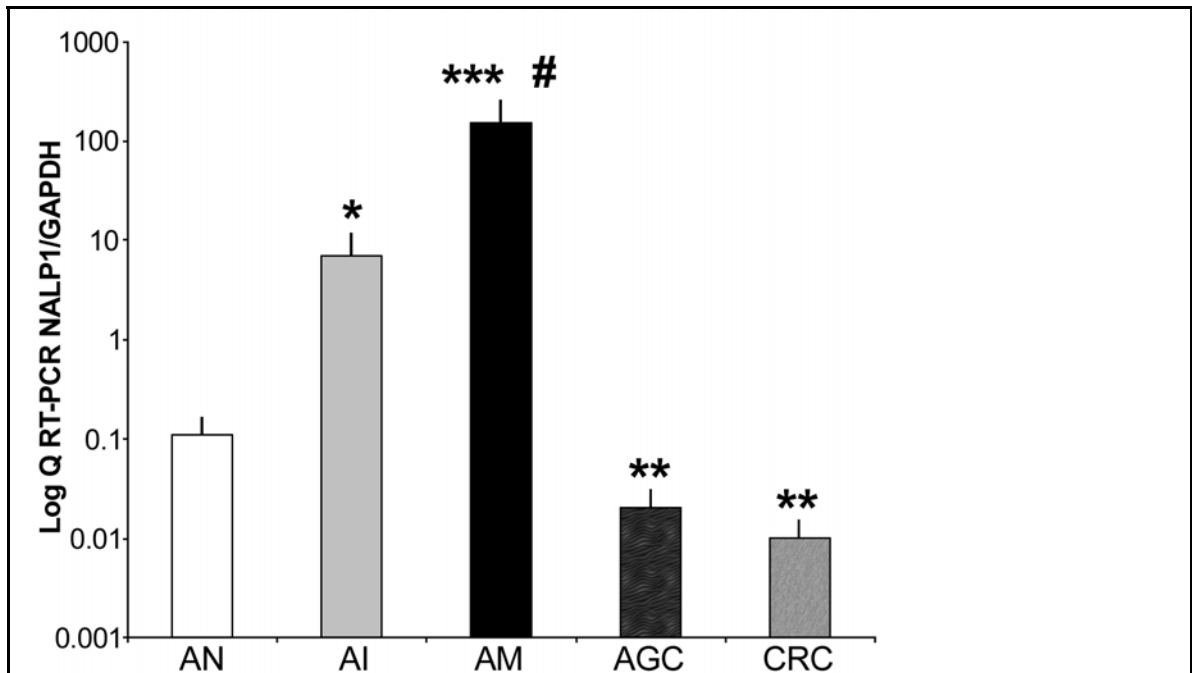
### 8. c. iv. *MTA1*:

*MTA1* is an estrogen-antagonistic breast cancer malignancy gene that has been used for the identification of progressive (metastatic) disease in a range of tumors including breast, hepatocellular, esophageal, gastric and colorectal carcinomas.<sup>75-79</sup> Message levels of *MTA1* were elevated 20–1000-fold, ( $p<0.01$ ) in the malignant APCs, GBC adenocarcinoids and colorectal adenocarcinomas compared to normal mucosa [Figure 8]. Both malignant appendiceal tumors and colorectal tumors had elevated levels of *MTA1* compared with incidental carcinoids. No differences in expression were noted between the incidental tumors and normal mucosa.



### 8. c. v. *NALP1*:

The apoptotic marker, *NALP1*, was over-expressed ~50–100-fold, ( $p<0.05$ ) in the incidentally-identified (“benign”) and malignant APCs compared to normal mucosa [Figure 9]. *NALP1* was significantly decreased ( $p<0.05$ ) in the GBC adenocarcinoids and colorectal adenocarcinomas compared to normal mucosa. In addition, malignant carcinoids had significantly elevated expression compared to all other tumor types.



**Figure 9.** Message levels of *NALP1* determined by Q RT-PCR.

Levels of *NALP1* were significantly over-expressed (~100x) in incidental (benign) APCs (AI) and in malignant APCs (AM; >1000x) compared to normal mucosa (AN). Levels were significantly decreased in APCs with GBC morphology (AGC; ~15x) and in colorectal cancer (CRC; ~1000x) samples compared to normal mucosa (AN). Malignant carcinoids had elevated *NALP1* levels compared to incidental carcinoids, GBC carcinoids and CRC tumors. ( $^{\#}p=0.05$ ,  $^{**}p=0.05$ ,  $^{*}p<0.01$ ,  $^{***}p<0.005$ ). Mean $\pm$ SEM.



#### ***8. d. Clinical relationship between levels of gene expression and appendiceal disease***

Two of the 25 patients included in this study were lost to follow-up; both of these patients belonged to the cohort of 16 patients with “incidental” tumors. None of the remaining 14 patients with incidental tumors for whom follow-up information was available were subsequently identified with lymph node or liver metastases (mean follow-up 113 months: range 8-372) [Table 1].

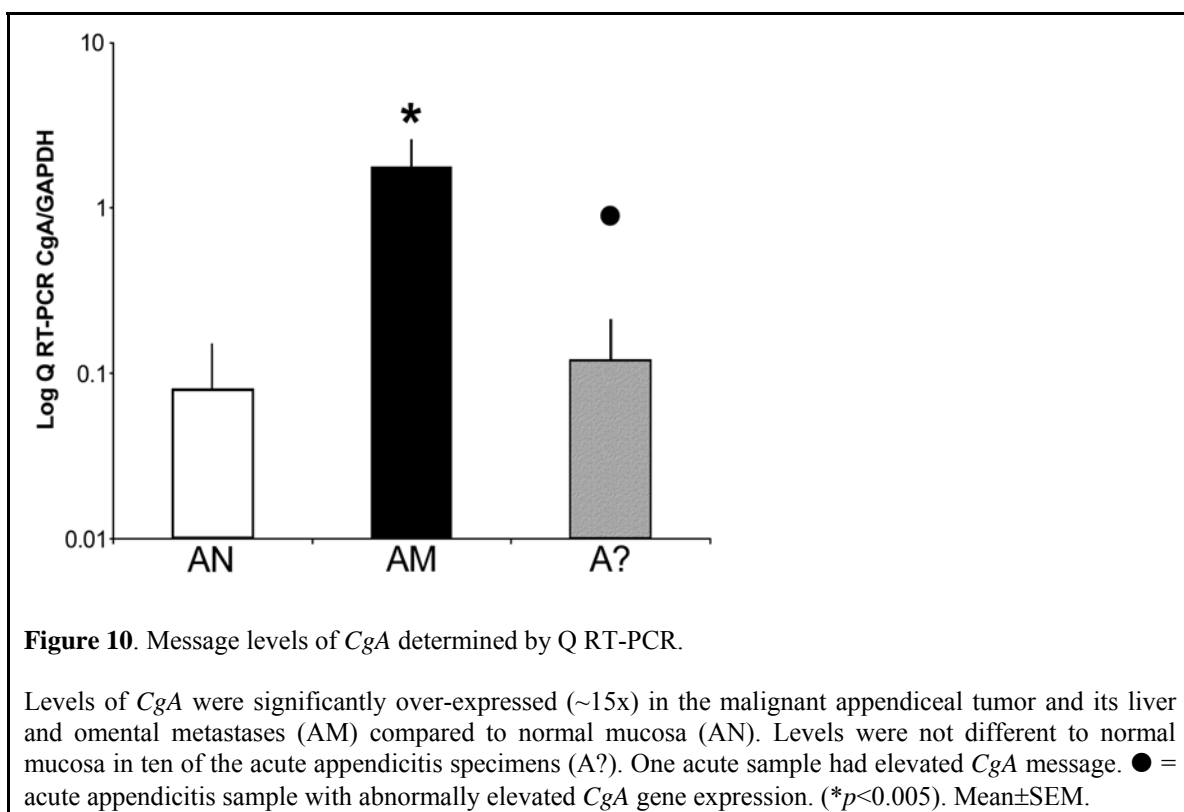
In the group of nine patients diagnosed with malignant tumors, one patient developed liver metastases (mean follow-up for this group was 199 months: range 33-468). The small number of patients precludes a robust statistical analysis of this data.

Pathologically, the “malignant group” tumors could be separated into tumors with local invasion ( $n=3$ ), tumors with lymph node metastases ( $n=5$ ) and a tumor with a liver metastasis ( $n=1$ ). An examination of gene expression levels in these categories demonstrated that four of the five candidate genes could be associated with lymph node or liver metastases. Thus levels of *CgA*, *NAPILI*, *MAGE-D2* and *MTA1* were ~100 fold higher in the tumors that had pathological evidence of metastases compared to APCs that were locally invasive. Interestingly, gene expression levels in the tumors classified as locally invasive were not different to the 16 patients with incidental tumors suggesting a threshold of expression may be required prior to the development of metastatic disease.

These data demonstrate that gene levels of four of the five markers are potentially clinically significant and that there is no overlap in gene expression levels between tumors that were classified as incidental (e.g. disease-free) and tumors classified as malignant (e.g. disease-specific).

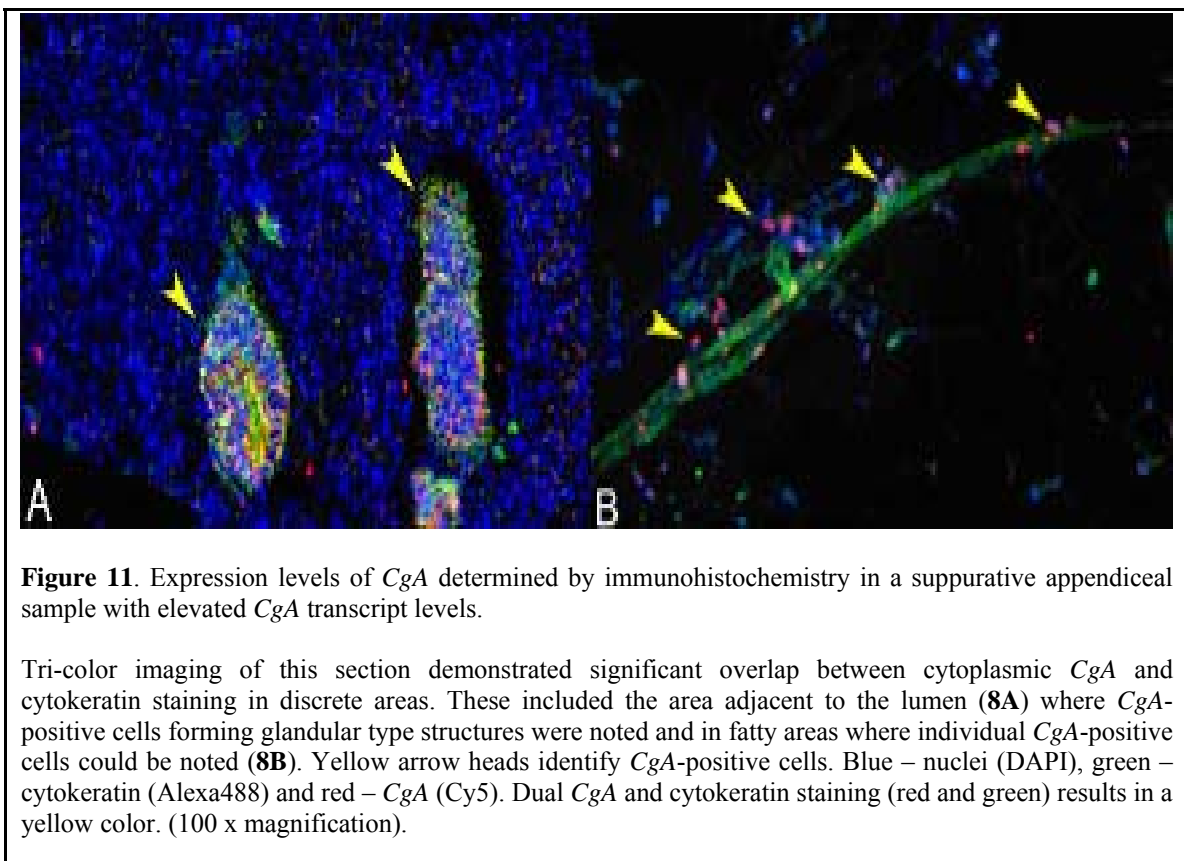
### 8. e. Prospective Q RT-PCR Analysis of Fresh Frozen Appendices

Levels of *CgA* were used to determine whether any covert appendiceal tumors could be identified in eleven prospectively collected surgical acute appendicitis cases. Levels were compared to normal appendiceal samples and to a highly malignant appendiceal tumor with liver and omental metastases. *CgA* levels were elevated in the positive controls compared with normal mucosa ( $p<0.0008$ ) and ten of the eleven appendicitis specimens ( $p<0.005$ ) [Figure 10].



Levels of *CgA* were low in appendicitis samples, except for one case (acute suppurative appendicitis with peri-appendicitis) that exhibited *CgA* levels at ~10x levels present in other tissues. This was significantly ( $p<0.02$ ) elevated compared to both the normal mucosa and other appendicitis specimens. Expression levels of the other four marker genes (*MAGE-D2*, *NAPILI*, *MTA1* and *NALP1*) were not elevated in this sample

and levels were not different to expression levels in the 16 “incidental” carcinoids (examined above). Staining of this appendiceal specimen demonstrated the presence of a cluster of *CgA*-immunopositivity [**Figure 11**]. This was absent in samples without elevated *CgA* gene expression. Based on these observations, it is plausible that one of the eleven surgically resected appendiceal specimens is worthy of consideration to be up-graded to a covert appendiceal tumor.



## 9. Discussion

These data demonstrate, using a Q RT-PCR approach in paraffin-embedded tissue, that malignant APCs, which like small intestinal carcinoids are derived from the EC cell, have elevated expression of *CgA*, *NAP1L1*, *MAGE-D2* and *MTA1* compared to incidentally identified APCs [Table 4].

Marker	AI	AM	GC	CRC
<b><i>CgA</i></b> (neuroendocrine)	↑	↑↑	↑	↔
<b><i>NALP1</i></b> (apoptosis)	↑	↑↑↑	↓	↓
<b><i>MTA1</i></b> (metastasis)	↔	↑↑↑	↑↑	↑↑↑
<b><i>MAGE-D2</i></b> (adhesion)	↔	↑↑↑	↑↑	↑↑↑
<b><i>NAP1L1</i></b> (mitosis)	↔	↑↑	↑	↔

**Table 4.** Summary of gene expression.

AI – incidental APC; AM – malignant APC; GC – goblet cell APC; CRC – colorectal carcinoma  
 ↑ - message levels elevated up to 10x compared to normal mucosa; ↑↑ - message levels elevated between 10x-100x; ↑↑↑ - message levels elevated >100x; ↓ - message levels decreased up to 10x; ↔ - no change

GBC adenocarcinoids, which are a mixed cell tumor type that also includes NE cells, also expressed elevated *CgA*, *NAP1L1*, *MAGE-D2* and *MTA1* compared to normal mucosa. These levels were not as elevated as in the malignant EC derived carcinoid tumors. Incidentally identified tumors, like overt malignant carcinoids, had elevated *CgA* and elevated *NALP1* expression. In contrast, adenocarcinoids had significantly decreased *NALP1* expression. The difference in *NALP1* expression (elevated in APCs, decreased in GBC adenocarcinoids) provides a molecular marker to differentiate between carcinoids

and adenocarcinoids of the appendix. *NAPILI* is the human equivalent of the yeast NAP-I protein, a histone-binding factor required for the maintenance of cumulative nucleosome formation *in vivo*.<sup>89</sup> Increased expression of *NAPILI* may be related to the progression of cell growth, because levels of both *NAPILI* mRNA and protein increase rapidly in conjunction with the induction of cellular proliferation in a T-lymphoid cell model.<sup>88</sup> In genome-wide profiling, *NAPILI* has been identified to be overexpressed in fetal liver compared with adult liver and in hepatoblastomas compared with nondiseased adult livers.<sup>73, 90</sup> Serological identification of antigens by recombinant expression cloning technology, which is used to search for genes whose products elicit antibody production in the patient, has identified *NAPILI* to be a potential serological antigen in a subset (<5%) of breast, renal, and colorectal cancer patients, but the mRNA study results were largely negative in these studies.<sup>91</sup> Messenger RNA levels of *NAPILI* were elevated in malignant APC tumors and in GBC adenocarcinoids compared to normal mucosa or incidentally identified lesions. Levels in colorectal adenocarcinomas were not different to normal mucosa. This confirms that *NAPILI* is a marker of appendiceal carcinoid malignancy.

*MAGE-D2* has been examined in the clinical setting by using high-density oligonucleotide DNA arrays and has been identified as a molecular marker to predict liver metastases from colorectal tumors.<sup>74</sup> It is overexpressed in >75% of primary colon tumors with metastases. Its function is still unknown, but its similarity to troponin indicates that it is involved in cell adhesion and increased expression is thought to facilitate the adhesion of cancer cells to vascular epithelium.<sup>74, 92</sup> The overexpression of *MAGE-D2* in malignant APCs, GBC adenocarcinoids demonstrates that assessment of

this marker has utility as a component of a panel for identifying and predicting the malignant and metastatic behavior of carcinoid tumors.

*MTA1* is a component of the nucleosome remodeling and histone deacetylation complex, which is associated with adenosine triphosphate-dependent chromatin remodeling and histone deacetylase activity.<sup>75</sup> Functionally, *MTA1* is also involved in the transcriptional repression of methylated DNA, and in breast tissue, *MTA1* represses estrogen receptor-mediated transcription and is therefore estrogen antagonistic.<sup>93, 94</sup> In breast tissue, the presence of estrogen receptors is usually associated with less aggressive tumors.<sup>95</sup> *In vitro* analysis of *MTA1* expression in estrogen receptor-positive cells is associated with increased proliferation and a more aggressive phenotype.<sup>94</sup> *MTA1* is normally expressed at low levels in various tissues but, like the cancer testes antigens, is more highly expressed in the testis.<sup>96</sup> On DNA arrays, *MTA1* is selectively overexpressed in metastatic prostate cancer compared with clinically localized prostate cancer and benign prostate tissue.<sup>97</sup> In a prostate cancer TMA, a strong relationship between *MTA1* expression and prostate cancer progression has been identified.<sup>97</sup> The close correlation between mRNA and protein levels of *MTA1* and the utility of this marker to identify progressive disease in a range of tumors indicate that this marker will be useful in identifying and predicting the metastatic behavior of carcinoid tumors. Both malignant appendiceal tumors and colorectal tumors had elevated levels of *MTA1* compared with incidental carcinoids. No differences in expression were noted between the incidental tumors and normal mucosa. This confirms that *MTA1* is a marker of carcinoid tumor metastasis.

Previous studies have not identified specific genetic differences in EC cell-derived appendiceal tumors compared to other appendiceal tumors or to normal mucosa. In one study, no mutations were identified in *K-ras*, *β-catenin*, or *DPC4* in GBC carcinoids and *p53* was not elevated, while another study determined that mucinous and non mucinous carcinomas of appendix had similar genetic alterations.<sup>59,98</sup> The current study, which uses a defined panel of biologically-relevant marker genes, can distinguish different NE tumor types found in the appendix.

The clinical relevance of this strategy is highlighted by the observation that none of the patients with low expression levels developed metastasis. Nevertheless the relatively short follow-up (113 months although follow-up in five of the 16 patients extended >19 years), indicates that at this stage a degree of caution is necessary in interpreting these results. Patients with high expression levels had pre-existing malignant disease or subsequently developed metastases irrespective of the length of follow-up. This group, however, was two decades older ( $p = 0.053$  versus patients with incidental tumors) than patients with incidental tumors, although the difference in age was not statistically significant. Clearly, a prospective study with longer follow-up in appropriate sex and age-matched patients is required to definitively evaluate the relationship between gene expression of these markers and disease progress in APCs.

While histological examination is useful in staging appendiceal disease, it is limited since a pattern-recognition technique is vulnerable when early cellular transformation events are occurring and can only broadly predict biological outcome once obvious changes are evident. The ability to identify at the molecular level gene regulators that govern proliferation and invasion has obvious potential advantages. In this

respect the objective quantification of gene expression levels, particularly genes with defined biological functions is of potential considerable clinical advantage. Thus, in patients where a carcinoid tumor of the appendix is identified and the need for further surgical intervention is uncertain since the criteria of tumor size, location and light microscopy are either inconsistent or provide ambiguous information, it is likely that the determination of gene expression may offer novel predictive information of considerable clinical relevance. Currently available information on which therapeutic strategy is based requires the exercise of clinical judgment - a commodity both quite variable and sometimes dubious in its application as opposed to objectively quantifiable molecular data.

In the current study, using a molecular PCR-based approach, *CgA* expression was detected in one of eleven histologically-negative fresh-frozen appendicitis samples. Light microscopic examination of tissue sections, (4 $\mu$ m thickness), by a pathologist (RLC) failed to identify a carcinoid tumor. Subsequent immunostaining of this section with anti-cytokeratin and anti-*CgA* followed by tyramide amplification of the *CgA* signal identified clusters of cells both adjacent to the lumen and within appendiceal peri serosal fat. The former appeared to have an epithelial morphology but were intensely *CgA*-positive. The latter were consistent with microcarcinoids. It is possible that injury or inflammation may be implicated in endocrine cell differentiation and that such events represent cytokine mediated phenomena.<sup>99</sup> Alternatively, such agents with well defined growth factor like bio active properties may cause appendiceal endocrine cell hyperplasia. The latter phenomenon has not been carefully examined in the appendix but is well-described in association with chronic bronchopulmonary inflammation.<sup>100, 101</sup> In addition chronic



atrophic gastritis is also associated with ECL cell hyperplasia and may well reflect a similar series of inflammation mediated events. It is note worthy that prolonged infection and chronicity are key requirements in such circumstances. If either of these two etiologies were responsible for the elevated *CgA* noted in our study, we would expect all samples from the eleven patients with suppurative appendicitis to express elevated levels of this marker. This was not the case. We therefore propose that the single patient with elevated *CgA* message and *CgA* protein expression is an authentic example of a covert appendiceal tumor detected using a molecular targeted strategy. Additional genetic examination of this specimen, using gene expression of *NAP1L1*, *MAGE-D2*, and *MTA1*, identified that levels of these markers were all within normal range. This serves to support the opinion that this specimen was non-malignant (no expression of malignancy-associated genes) and could potentially be categorized as an incidental non-malignant APC tumor.

This observation suggests that in acute appendiceal samples obtained at surgery, covert carcinoid tumor not readily identifiable by standard light microscopy can be identified using a molecular screen. Indeed, our previous demonstration that approximately ~25% of histologically normal lymph nodes in small bowel carcinoid resections are *CgA*-PCR-positive (indicative of covert metastasis) suggests that this technique will be of similar utility in the identification of covert appendiceal NE tumors.<sup>102</sup> In general, the detection rate for APCs using standard histological techniques in appendectomy samples is ~1%.<sup>42</sup> Our study, using a more sensitive PCR molecular genetic approach, suggests that this may well be higher.

RNA isolation from paraffin-blocks is becoming an acceptable method for examining gene expression. In the current study, RNA was isolated from all samples and the genes of interest were readily amplified. This confirms the utility of this technique in APC samples as has been previously demonstrated for other tumor types and tissue samples including Barrett's esophageal adenocarcinomas and breast tumors.<sup>82, 83, 103</sup> Furthermore, CgA transcript levels from these paraffin blocks could be related to protein expression levels identified on a tissue microarray.<sup>102</sup> Correlating CgA transcript from the current study with protein levels of CgA measured by AQUA in the same appendiceal tumors demonstrated these were significantly related:  $R^2 = 0.40$ ,  $p < 0.03$ . Absence of an absolute correlation may represent a degree of RNA degradation but is more likely to be due to differential processing of transcript.<sup>104</sup>

## ***10. Summary***

Carcinoid tumors of the gastrointestinal tract are relatively rare compared with their adenocarcinomatous counterparts. Nevertheless, they may display similarly aggressive behavior. Timely and accurate diagnosis is frequently absent because symptoms and signs may be vague and nonspecific and misconstrued as irritable bowel syndrome, asthma, or perimenopausal symptoms or part of an anxiety or food allergy response. Importantly, the "classical" carcinoid syndrome is expressed in relatively few instances. Because each lesion is composed of its own distinct NE cell(s), depending on the organ of origin, each tumor behaves as a different biological entity that requires a site-specific therapeutic approach. However, common to all carcinoid tumors is the high percentage of coexisting noncarcinoid tumors and multicentricity, warranting a meticulous evaluation

during diagnosis and treatment. To facilitate improvements in diagnosis and therapy, it is imperative to elucidate the NE cell type involved in tumorigenesis, define their growth regulation, characterize their secretory products, and establish the molecular basis of the individual tumors. The need to define a plasma or genetic marker to predict or diagnose early lesions is paramount.

Our data demonstrate over-expression of *CgA* and *NALP1* in APCs, over-expression of *NAPILI*, *MAGE-D2* and *MTA1* in malignant APCs and mixed cell (GBC) adenocarcinoids, and decreased expression of *NALP1* in the latter tumor type. We therefore propose that this evaluation supports the utility of the measurement of such biomarkers to differentiate appendiceal tumor types both in paraffin-embedded and fresh frozen samples. The ability to identify occult carcinoid tissue by *CgA* expression with such amplified sensitivity also indicates that this technique may have application in the detection of appendiceal tumors or their metastasis that cannot be identified by conventional pathological techniques. The implications for altering staging and hence therapeutic strategy are of clear clinical relevance.

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